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Full Length Research Paper

Expression and clinical significance of matrix metalloproteinase 9 (MMP9) papillary thyroid carcinomas

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Matrix metalloproteinases (MMPs) play a crucial role in tumor invasion and metastasis. There have been only a few studies on the MMP expression in thyroid carcinomas. Therefore, we investigated the MMP9 expression in 66 papillary thyroid carcinomas (PTC) using immunohistochemistry and quantitative reverse transcription-polymerase chain reaction (qRT-PCR) on protein level and mRNA level, respectively. We also examined the correlations between the immunohistochemical scores and several clinicopathological parameters. The results demonstrated that MMP9 was significantly up-regulated in cancer tissue in comparison to begnin thyoid tumor tissue on mRNA level ($P < 0.05$). On protein level, MMP9 was also highly increased in cancer tissue in comparison to begnin thyoid tumor. It is found that high expression MMP9 proteins significantly correlated with large tumor size, presence of lymph node metastasis, Union Internationale Control Cancer (UICC) stage. These data suggest that MMP9 proteins are increased in tumors cells of PTC and that it plays an important role in the invasion and metastasis of PTC.

Key words: Thyroid carcinomas, matrix metalloproteinase 9 (MMP9), quantitative reverse transcription-polymerase chain reaction (qRT-PCR), immunohistochemistry, papillary thyroid carcinomas.

INTRODUCTION

Thyroid carcinoma is the most common malignancy of the endocrine system and accounts for approximately 1% of all newly diagnosed cancer (Guang et al, 2011., Chen et al., 2011; Hundahl et al., 2000; Roskies et al., 2012). Thyroid cancer can be divided into papillary thyroid carcinomas (PTC), follicular, medullary, or anaplastic histological types. PTC is the most common type of thyroid cancer and accounts for more than 83% of all thyroid malignancies (Brown et al., 2011; Dal Maso et al., 2009). Hence, it is important to understand molecular mechanisms responsible for PTC development, progression and metastasis, and in turn, develop novel strategies for the early detection, prevention, and treatment of thyroid cancer.

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Tumor invasion is a cascade of sequential events that involves detachment of malignant cells from their site of origin and invasion through the surrounding stroma into lymphovascular channels (Hagemann et al., 2012). All these steps are associated with extracellular matrix (ECM) degradation, and the proteolytic breakdown of major ECM components requires specific proteases. Matrix metalloproteinases (MMPs) are proteolytic enzymes that degrade ECM components at neutral pH (Salamonsen et al., 1996; Chambers et al., 1997). Numerous studies have demonstrated that individual MMPs play crucial roles in tumor invasion and metastasis (Parson et al., 1997; Westermarck et al., 1999; Curran et al., 1997; Yu et al, 2012). Expression of MMP-2 is considered especially important as an indicator of tumor aggressiveness in a variety of neoplasms (Kameyama et al., 1996; Demeure et al., 1992). The high frequency at

which MMP3 are detected at mRNA and/or protein levels in invasive tumor cell lines and various human carcinomas suggests that MMPs are implicated in invasion and metastasis (Liotta et al., 1991; Tryggvason et al., 1993). Recent study showed that MMP9 were over-expressed in gastric tumours compared with normal tissue and it may act together to increase carcinogenesis and the progression, invasion and metastasis of gastric carcinoma (Yang et al., 2011). However, little studies reported the expression of other MMPs including MMP9 in PTC.

In the present study, we examined the expression levels of MMP9 in human PTC on mRNA level and protein level by quantitative reverse transcription-polymerase chain reaction (qRT-PCR) and immunohistochemistry, respectively. We also tested the correlations between the immunohistochemical scores and several clinicopathological parameters to find out the association between MMP9 and PTC.

MATERIALS AND METHODS

Clinical samples and patients

Fresh tissue samples of thyroid were obtained from patients with primary papillary carcinoma (66 cases), 20 cases of thyroid adenoma, 20 cases of multi-nodular goiters, and 10 cases of normal thyroid who underwent surgery in the Department of Thyroid Surgery at The First Affiliated Hospital of Jilin University between January and September, 2009 to March, 2011. The patients with PTC ranged in age from 19 to 68 years (mean ± SD, 40.2 ± 0.6 years), which included 31 male (46.9%) and 35 female (53.1%), 37 patients were younger than 45 years and 29 patients were older than 45 years.

Diagnosis and staging of PTC was performed according to the Union Internationale Control Cancer (UICC) classification (Tumor-Node-Metastasis, 1992). Stages I and II were found in 40 (61%) patients, and III and IV in 26 (39%) patients. Surgical specimens were fixed with 10% buffered formalin, and paraffin sections were stained with Hematoxylin and eosin (H&E). This study was approved by the ethics committee of Jilin hospital and an informed consent was obtained from all participants.

qPCR

Total RNA was isolated from frozen thyroid tissue using TRIZOL reagent (Invitrogen, USA) according to the manufacturer's protocol and as described in the online supplement. The purity and concentration of RNA were determined using a dual beam ultraviolet (UV) spectrophotometer (Eppendorf AG, Hamburg, Germany). Using mRNA as template, single-stranded cDNAs were generated by Superscript II reverse transcriptase (Invitrogen) according to the manufacturer's directions. The MMP9 primer sequences are as follows: Sense prime: 5′- TGGTGTGCCCTGGAACTCA-3′; Anti-sense prime: 5′- TGGTGTGCCCTGGAACTCA -3′.

The qPCR conditions are as follows: an initial 95°C for 3 min and followed by 40 cycles of 95°C for 15 s and 55°C for 1 min. A dissociation curve was established after each PCR in order to verify amplification specificity. The integrity of the MMP9 and the efficiency of qPCR in each sample were confirmed by the endogenous control U6 small RNA. Negative control experiments were set without cDNA template. The relative quantification of each MMP9 was presented as the fold change after normalized to the U6 RNA for the Equation $2^{-\Delta\Delta Ct}$ in the Rotor-Gene 6000 Series Software 1.7 (Qiagen), where $\Delta Ct = (CtmiRNA - CtU6)$. Expression of MMP9 in lesions was compared to the normal tissues using a formula:

$\Delta\Delta Ct$ = (Ct tumor MMP9 - Ct tumor U6) - (Ct normal MMP9 - Ct normal U6).

Immunohistochemistry

To detect expression and localization of MMP9 in thyroid tissue, immunohistochemical was performed using SP reagent kit (Tiangen, China) according to the manufacturer's protocol and as described in the online supplement.

The degree of immunoreactivity was assessed similarly to the system described by Campo et al. (1992). The intensity of the staining was graded as 1+, 2+ or 3+. In cases with variable staining intensities, the most common pattern was recorded. The extent of staining was considered as 1+ if the number of reactive cells was less than 25%, 2+ (25 ± 75% reactive cells), and 3+ (more than 75%). Cases with 0 or 1+ staining were classified as negative, and cases with 2+ or 3+ staining were classified as positive. The final score was obtained in each case by adding the intensity to the extent score. Immunoreactivity was then classified according to the combined score as weak (scores 1 and 2, +), moderate (3 and 4, ++), or strong (5 and higher, +++). All slides were scored independently by two observers (M.B.R. and O.Y.). Five cases with discordant results were reevaluated to obtain agreement.

Statistics analysis

To calculate the statistical differences between thyroid carcinomas and benign tumor, the statistical package SAS 6.12 (USA) was used for all analyses. Methods were Pearson Chi-square test and Pearson correlation analysis. Student's *t*-test was used to determine the significance of differences between the groups. All values were expressed as mean ± SD. In general, p-values less than 0.05 were considered statistically significant.

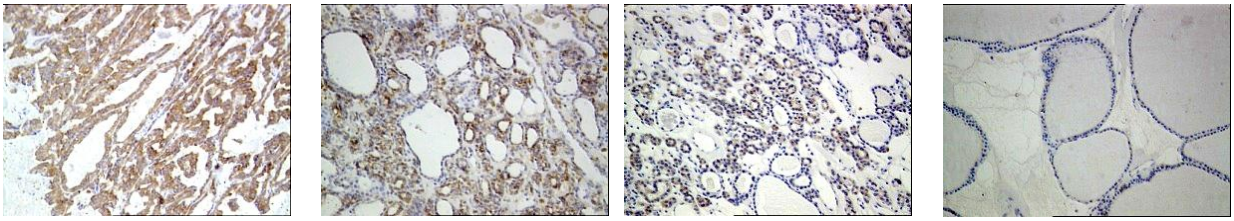
RESULTS

qRT-PCR analysis of mRNA expression of MMP9

In order to detect the mRNA expression of MMP9 in patients with thyroid carcinoma, qPCR was conducted. As shown in Table1, MMP9 was significantly over-expressed in thyroid papillary cancers compared to the thyroid adenoma, normal thyroid tissues and multinodular goiters ($P < 0.05$) (Table 1). However, there was no significant difference among the thyroid adenoma, normal thyroid tissues and multinodular goiters (Table 1).

Immunohistochemistry analysis protein expression of MMP9

In order to further confirm MMP9 protein expression, immunohistochemistry was conducted. As shown in Figure 1, thyroid adenoma and multinodular goiters thyroid,



(A) (B) (C) (D)

Figure 1. The expression of MMP9 protein by immunohistochemical staining at thyroid carcinoma tissue, thyroid adenoma, normal thyroid tissues and multinodular goiters tissue. A, Thyroid carcinoma tissue; B, thyroid adenoma; C, multinodular goiters; D, normal thyroid tissues.

Table 1. Expression of MMP9 mRNA in thyroid carcinoma tissue, thyroid adenoma, normal thyroid tissues and multinodular goiters.

Group	n	$\bar{x}$ ± s (copy/μg RNA)
PTC	66	577186.97 ± 48354.25^{A}
Normal tissue	10	20428.14 ± 143315.84^{B}
Thyroid adenoma	20	21976.98 ± 167021.72^{B}
Goiters	20	23057.39 ± 143315.68^{B}

Different marks represent the significant difference at p < 0.05.

Table 2. Expression of MMP9 in thyroid carcinoma tissue, thyroid adenoma, normal thyroid tissues and multinodular goiters by immunohistochemical staining.

Group	n	MMP9		
		-	+	(%)
PTC	66	5	61	92.4
Thyroid adenoma	20	14	6	30.0
Multinodular goiters	20	18	2	10.0
Normal tissue	10	10	0	0

tumor cells and stromal fibroblasts had little immunostained in any of the cases, Also, no staining was seen in the control normal samples. MMP9 was immuno-localized mainly to the carcinoma cells. Table 2 showed that there were significantly differences ($P < 0.05$). MMP9 expression between thyroid carcinoma and thyroid adenoma, and normal thyroid tissues and multinodular goiters ($P < 0.05$); MMP9 protein expression levels in thyroid carcinoma group were significantly up-regulate compared to other group ($P < 0.05$),

Associations of clinical and pathological variable and MMP9 expression in PTC

In this study, we analyzed the relationship between MMP9 protein expression and clinical and pathological variables, including gender, mean age, UICC stage and lymph node degree in PTC, using Pearson Chi-square (χ^2) test. We found significant associations between MMP9 protein expression and UICC stage, tumor size and lymph node metastasis ($P < 0.05$, Table 3). MMP9 up-expression was significantly associated with UICC stage ($P = 0.006$), being observed more frequently in cases Stages III to IV (100%) than in cases Stages I to II (87.75%). However, MMP9 protein expression had no significant associations with gender and age.

DISCUSSION

In the present study, we chose to study the MMP9 gene and to assess its expression and association in different thyroid tissues. qPCR experiment verified that MMP9 production was increased on the mRNA level of thyroid carcinoma when compared to thyroid adenoma and normal thyroid tissues and multinodular goiters ($P < 0.05$), On protein level, MMP9 expression production in thyroid carcinoma group were significantly up-regulate compared to other group ($P < 0.05$) using immunohisto-chemistry. Furthermore, we identified significant asso-ciations between MMP9 protein expression and UICC stage and lymph node metastasis ($P < 0.05$). The current study provides important data on the involvement of MMP9 in the pathogenesis of thyroid carcinoma.

The current studies have demonstrated that the production level of MMP9 is remarkably higher in the carcinomas than in the non-carcinoma tissues. The enhanced production of MMP9 protein have been reported in many human carcinomas, including stomach (Nomura et al., 1996), breast (Ueno et al., 1997), lung (Tokuraku et al., 1995) and endometrial carcinomas (Yu et al., 2012). Moreover, some studies showed that the production levels of MMP9 in the carcinoma tissue are negligible due to unique of thyroid carcinomas (Nakamura et al., 1999), which did not comply with our results that the production level of MMP9 is remarkably

Table 3. The associations between MMP9 expression and clinicopathological features of thyroid papillary cancers.

Clinicopathological feature	n	MMP-9			
		-	+	χ^2	P-value
Thyroid adenoma	20	14	6	1 vs 2:38.969	1 vs 2:0.001
Multinodular goiters	20	18	2	1 vs 3:51.802	1 vs 3:0.001
Normal tissues	10	10	0	2 vs 3:3.805	2 vs 3:0.05
PTC	66	5	61		
Lymph nodes					
No	12	2	10	1.906	0.167
Yes	54	3	51		
Tumor size (cm)					
≤1.0	17	3	14	11.553	0.001
>1.0	49	2	47		
UICC stage					
I - II	40	5	35	7.470	0.006
III - IV	26	0	26		
Gender					
Male	31	1	30	1.826	0.177
Female	35	4	31		
Age					
<45	37	5	32	0.076	0.003
≥45	29	0	29		

increased in thyroid carcinomas; the reason might be that tissue sample of thyroid carcinomas was different form our study or that study method was different from our method. In this study, we collected enough sample, mRNA level and protein level was studied by qPCR and immunohistochemistry, respectively, therefore, our result might be more reasonable and concise.

In conclusion, the present study demonstrated that MMP9 expression was significantly up-regulated in thyroid carcinoma tissue in comparison to the thyroid adenoma and normal thyroid tissues and multinodular goiters ($P < 0.05$). These results implied that MMPs play a key role in tumor invasion and metastasis, and suggested that increased MMP9 expression might be a useful diagnostic marker and might also become a potential target in the treatment of thyroid carcinoma.

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